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Anaerobic Oxidation of Fatty Acids and Alkenes by the Hyperthermophilic Sulfate-Reducing Archaeon *Archaeoglobus fulgidus*▽†

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Archaeoglobus fulgidus **oxidizes fatty acids (C_4 to C_{18}) and *n*-alk-1-enes ($C_{12:1}$ to $C_{21:1}$) in the presence of thiosulfate as a terminal electron acceptor. End products of metabolism were CO_2 and sulfide. Growth on perdeuterated hexadecene yielded C_{15}- to C_{17}-labeled fatty acids as metabolites, thus confirming the ability of *A. fulgidus* to oxidize alkyl chains.**

Many studies have shown that under anaerobiosis, hydrocarbon oxidation can be coupled to sulfate reduction (28), and several sulfate-reducing bacteria have been reported to oxidize *n*-alkanes and/or *n*-alkenes (13, 15). Most of these strains are mesophilic, except *Desulfothermus naphthae* strain TD3, which oxidizes alkanes at 55 to 65°C (23). However, in deep hot environments (e.g., oil reservoirs), it has been established that oil biodegradation could occur at temperatures up to 85 to 90°C (3). Until now, few hyperthermophilic sulfate-reducing microorganisms growing at temperatures higher than 80°C have been isolated from oil field environments. They include the genera *Desulfotomaculum*, *Thermodesulfobacterium*, and *Archaeoglobus* (20), but these microorganisms have never been tested for their ability to oxidize hydrocarbons. Among the *Archaea*, members of the genus *Archaeoglobus* are well represented in deep environments and quite widespread in marine (e.g., hydrothermal vents) and terrestrial (hydrothermal systems and oil reservoirs) hot environments (5, 6, 16, 19, 26, 27). Interestingly, the analysis of the complete genome of *Archaeoglobus fulgidus* strain VC-16 revealed the presence of β-oxidation genes (18). However, its ability to degrade a variety of hydrocarbons and organic acids has been hypothesized (18) but never demonstrated. In the present study, experiments were conducted to elucidate whether *A. fulgidus* VC-16 can oxidize *n*-alkanes or more oxidized compounds (*n*-alkenes and fatty acids) in the presence of thiosulfate or sulfate as a terminal electron acceptor.

Growth of *A. fulgidus* on long alkyl chains (fatty acids, *n*-alkanes, and *n*-alk-1-enes). *Archaeoglobus fulgidus* strain VC-16 (DSM 4304), isolated from a terrestrial heated sea floor at Vulcano, Italy (27), was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). This strain was cultivated on medium described by Zellner et al. (30) and modified as followed (g per liter unless indicated): NH_4Cl, 0.3; KH_2PO_4, 0.3; K_2HPO_4, 0.3; KCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; NaCl, 18; $Na_2S_2O_3$, 2.37 (15 mM); yeast extract, 0.1; $Fe_2SO_4 \cdot 7H_2O$, 1.42 mg; $NiSO_4 \cdot 6H_2O$, 1.6 mg; $Na_2WO_4 \cdot 2H_2O$, 38 μg; $Na_2SeO_3 \cdot 5H_2O$, 3 μg; L-cysteine hydrochloride, 0.5; resazurin, 1 mg; and trace element solution (4), 10 ml · liter^{-1}. The pH of the medium was adjusted to 7.0. The medium was prepared anoxically, and the headspace of the tubes and bottles was filled with N_2-CO_2 (4:1). Following sterilization, the medium was amended with $Na_2S \cdot 9H_2O$ (0.4 g · liter^{-1}), $NaHCO_3$ (2.0 g · liter^{-1}), $MgCl_2 \cdot 6H_2O$ (3.0 g · liter^{-1}), and 10 ml · liter^{-1} of a vitamin solution (29). Cultures were inoculated at 10% (vol/vol). For growth tests with hydrocarbons, a preculture on octanoate was used as an inoculum. The inoculum was washed with substrate-free medium and concentrated four times before use.

A. fulgidus was cultivated with fatty acids (2 mM), *n*-alkanes (1.2 mM), or *n*-alk-1-enes (1.2 mM) as an energy source. Cultures were grown at 70°C in tubes (Bellco) containing 10 ml of medium and sealed with butyl rubber stoppers. Tubes containing hydrocarbons were incubated upside down. Anaerobic oxidation of substrates was followed by measurement of dissolved sulfide production (8) and thiosulfate or sulfate consumption (ionic chromatography after sulfide removal by precipitation with zinc carbonate [50 g · liter^{-1}]). All cultures were performed in triplicate and compared to control cultures without substrate.

A. fulgidus was able to grow on a wide range of short- to long-chain fatty acids: butyrate, valerate, octanoate, nonanoate, palmitate, and stearate (results not shown). Sulfide production with fatty acids was in the range of 10.5 to 14.2 mM

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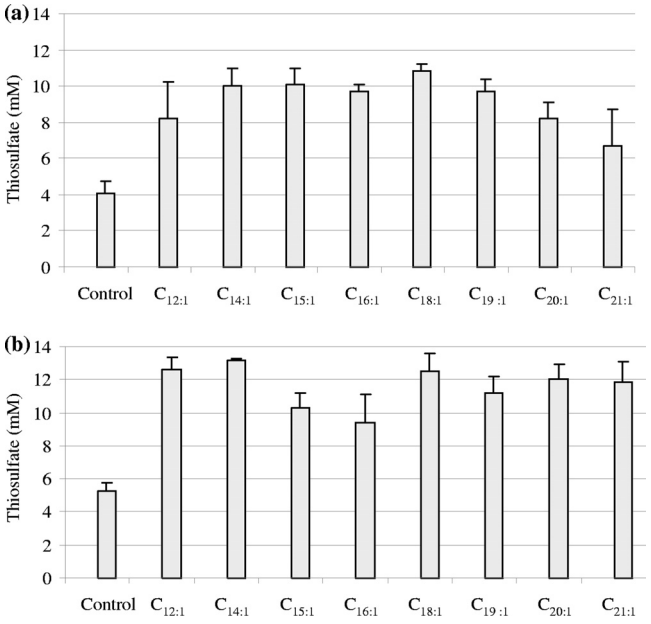

FIG. 1. Thiosulfate consumption after 24 weeks of incubation at 70°C of *A. fulgidus* strain VC-16 with C_{12} to C_{21} *n*-alk-1-enes. (a) First cultures; (b) corresponding subcultures. Control cultures were incubated without alkenes at the same temperature.

(soluble sulfide) after 7 weeks of incubation, whereas sulfide production in control cultures without a substrate reached only 1.8 mM. Although growth of *A. fulgidus* (DSM 8774) on short-chain fatty acids, such as valerate, in the presence of hydrogen has already been suggested (5), this is the first demonstration that the hyperthermophilic, sulfate-reducing archaeon *A. fulgidus* is able to use valerate but also longer-chain fatty acids (C_8 to C_{18}) as the sole energy source. Interestingly, the same ability was reported for its close hyperthermophilic relative *Geoglobus ahangari*, known to grow exclusively by reducing Fe(III) (17a).

The growth of *Archaeoglobus fulgidus* on some aliphatic saturated (dodecane, hexadecane, and pristane) and unsaturated (dodec-1-ene and hexadec-1-ene) hydrocarbons was also tested. After 1 month of incubation, based on soluble sulfide production and thiosulfate consumption, alkenes but not alkanes were shown to be oxidized (data not reported). Growth of *A. fulgidus* on individual alk-1-ene was further tested with compounds ranging from C_{12} to C_{21}. After 24 weeks of incubation, thiosulfate reduction to sulfide appeared clearly higher in cultures with alkenes than in control cultures incubated under the same temperature condition without a substrate (Fig. 1a). Moreover, all these cultures could be subcultured on the corresponding alkenes, thus proving efficient growth of *A. fulgidus* on these substrates (Fig. 1b). This demonstrated that *A. fulgidus* strain VC-16 is able to grow on C_{12} to C_{21} *n*-alk-1-enes as a unique source of energy. Growth of *A. fulgidus* on *n*-alk-1-enes was also demonstrated in the presence of sulfate (15 mM) instead of thiosulfate as a terminal electron acceptor.

Quantitative degradation of hexadec-1-ene. Quantitative growth experiments were carried out in tubes sealed with Teflon-coated rubber stoppers (West Pharmaceutical Services) containing 15 ml of medium and 5.5 μl of hexadec-1-ene (1.27 mM). Total sulfide production was determined after alkalinization of the culture by adding 0.3 ml KOH (10 M). The hexadecene concentration was determined by gas chromatography after extraction with heptane using pristane as an internal standard. Three replicate assays together with three biotic (without hydrocarbon but inoculated) and three abiotic (with hydrocarbon but not inoculated) controls were incubated for 17 weeks under the same temperature condition before analysis. Growth of *A. fulgidus* was demonstrated through the consumption of 0.17 mM hexadec-1-ene and the simultaneous production of 4 mM sulfide. Proportionally, 23.52 mM sulfide would have been produced from 1 mM hexadec-1-ene, which is close to the values expected from the following equation:

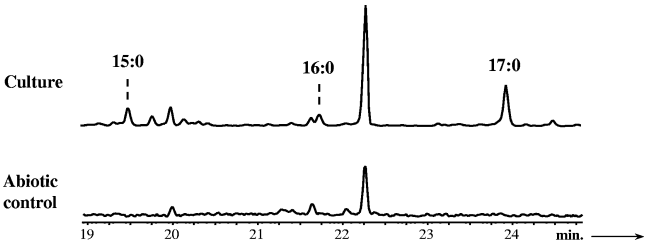

FIG. 2. Partial mass chromatograms (*m/z* 76 + 119 + 135) of total cellular fatty acids (silylated) of a culture of *A. fulgidus* strain VC-16 incubated with d_{32}-hexadec-1-ene at 70°C (top) and of a corresponding incubated abiotic control (noninoculated with hydrocarbon, bottom).

$$C_{16}H_{32} + 12S_2O_3^{2-} \rightarrow 16CO_2 + 24S^{2-} + 24H^+ + 4H_2O$$

Hydrocarbon depletion and sulfide production were not observed in incubated abiotic controls.

Identification of perdeuterated hexadecene-derived fatty acids. *A. fulgidus* was grown in 450 ml of anoxic medium with labeled hexadecene (perdeuterated d_{32}-hexadec-1-ene, 0.55 mM, synthesized from perdeuterated palmitic acid [Eurisotop] as described by Grossi et al. [14]). Cells were collected by filtration through glass microfiber filters (GF/B; Whatman) and treated with 1 M KOH in methanol-water (1:1 [vol/vol]). Saponifiable (acid) lipids were extracted from the acidified solution (9). Extracts were silylated [bis(trimethylsilyl)trifluoroacetanamide-pyridine (1:1, vol/vol)] and analyzed by gas chromatography-mass spectrometry (GC-MS) using a Finnigan Voyager MD800 mass spectrometer coupled to a 6890HP gas chromatograph equipped with a DB5MS capillary column (30 m by 0.25 mm; film thickness, 0.25 μm). Growth of *A. fulgidus* on d_{32}-hexadec-1-ene yielded deuterated fatty acids (Fig. 2 and 3). Isotopomers which differed by one or two deuterium atoms were formed but did not yield distinct GC peaks. Structural identification of the main isotopomers (d_{29}-15:0, d_{29}-16:0, and d_{31}-17:0 fatty acids) was achieved by careful selection of mass spectra using mass chromatograms of selected ions and by comparison with previously reported mass spectra (7, 9). The number of deuterium atoms in each fatty acid was deduced from the molecular ion ($M^{+\bullet}$) and from the ion corresponding to the loss of one methyl from the TMS group (M-15). These deuterated fatty acids were not detected in incubated abiotic controls (with hydrocarbon but not inoculated [Fig. 2]), further demonstrating that *A. fulgidus* strain VC-16 is able to oxidize *n*-alkenes anaerobically.

Previous studies have reported the biodegradation of *n*-alkenes by mesophilic sulfate-reducing bacteria (namely, strains

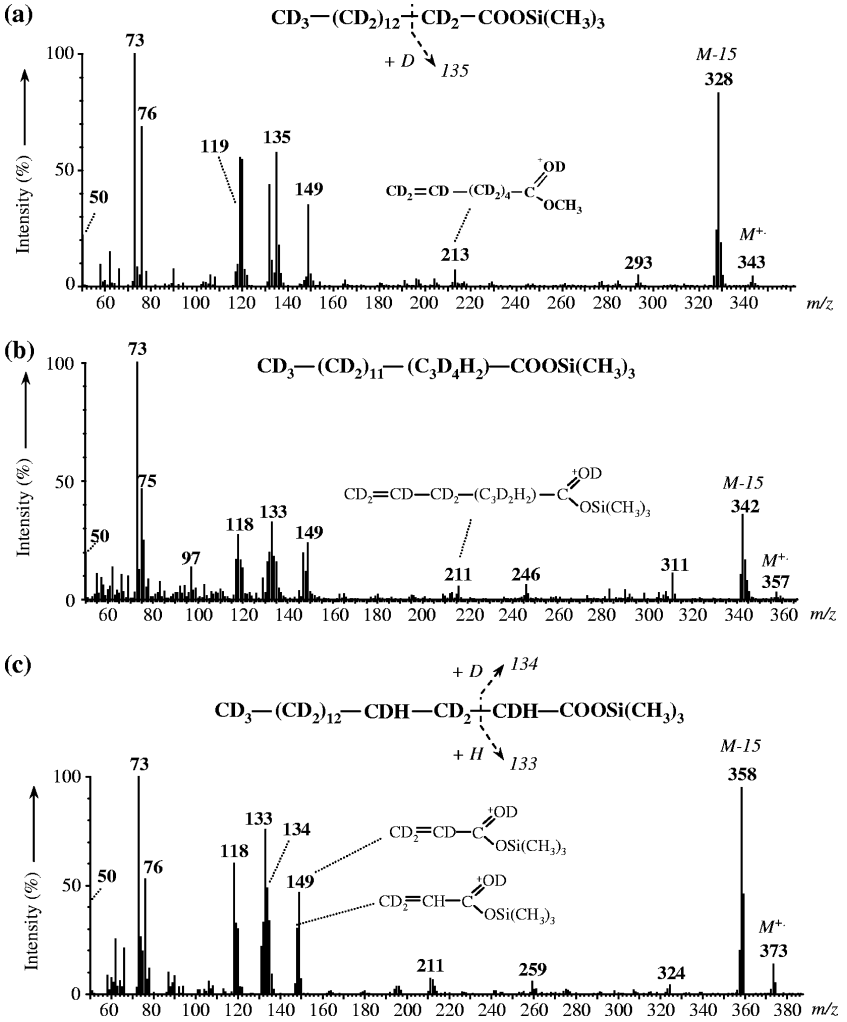

FIG. 3. Mass spectra and tentative structure of silylated d_{29}-pentadecanoic acid (a), d_{29}-hexadecanoic acid (b), or d_{31}-heptadecanoic acid (c) from a culture of *A. fulgidus* strain VC-16 incubated with d_{32}-hexadec-1-ene.

Hxd3 [1], Pnd3 [2], AK-01 [25], *Desulfatibacillum aliphaticivorans* strain CV2803 [11], *Desulfatibacillum alkenivorans* strain PF2803 [12], and *Desulfatiferula olefinivorans* strain LM2801 [10]). To our knowledge, this is the first report of the anaerobic oxidation of hydrocarbons, specifically *n*-alkenes, by a member of the domain *Archaea* and at a temperature as high as 70°C. Different initial reactions of anaerobic alk-1-ene oxidation by *D. aliphaticivorans* strain CV2803 have been proposed (14). This bacterium oxidized alkenes into either branched (methyl- or ethyl-) or linear fatty acids by addition of organic carbon or by hydroxylation of the double bond, respectively. In the present case, the formation of d_{29}-16:0 fatty acid from d_{32}-hexadec-1-ene may suggest that *A. fulgidus* also hydroxylated the alkene double bond at C-1. However, the loss of three deuterium atoms between the alkene substrate and the corresponding fatty acid (Fig. 3b) is not in agreement with the results obtained with *D. aliphaticivorans* strain CV2803, which induced the loss of only two deuterium atoms for the same oxidation step (14). The present results may be explained either by hydrogen/deuterium exchanges at high temperature and/or by distinct mechanisms of alk-1-ene oxidation between *A. fulgidus* strain VC-16 and *D. aliphaticivorans* strain CV2803. The involvement of a distinct mechanism in *A. fulgidus* strain VC-16 is further suggested by the formation of C-odd d_{29}-15:0 and d_{31}-17:0 fatty acids, which could not result from chain shortening (i.e., β-oxidation) or elongation of d_{29}-16:0 fatty acid. The formation of C-even and C-odd fatty acids from C-odd and C-even alkanes, respectively, has been demonstrated in the mesophilic sulfate-reducing bacterium strain Hxd3 (24). The mechanism proposed includes subterminal carboxylation with inorganic carbon at the C-3 position of the alkane and elimination of the two adjacent terminal carbon atoms, resulting in a fatty acid one carbon shorter than the original alkane but which can subsequently be elongated (24). A similar mechanism could explain the transformation of d_{32}-hexadec-1-ene to d_{29}-15:0 fatty acid by *A. fulgidus* strain VC-16 but would not support the presence of two additional deuterium atoms in d_{31}-17:0 fatty acid (Fig. 3c). Alternative mechanisms can be envisaged for the oxidation of alk-1-enes by strain VC-16, including the addition of an organic carbon unit distinct from the one involved in alk-1-ene oxidation by *D. aliphaticivorans* strain CV2803 (14) or the epoxidation of the

alkene double bond. These speculative mechanisms still require further investigation.

Enzymes involved in the activation of alkenes have not yet been defined. Johnson et al. (17) isolated and characterized a Mo-Fe-S-containing enzyme from *Azoarcus* sp. strain EB1 that was able to mediate, in the absence of oxygen, the hydroxylation of a branched alkene. This enzyme is a heterotrimer composed of a molybdopterin-binding subunit (EbdA), a [4Fe-4S] cluster binding subunit (EbdB), and a membrane anchor subunit that would bind a b-type heme. In addition to these structural genes, the operon would also include a chaperonin-like protein-encoding gene (*ebdD*) (see Fig. S4 in the supplemental material) (21). Genome analysis of *A. fulgidus* reveals the presence of a gene cluster encoding a molybdopterin oxidoreductase, exhibiting significant homology with the *ebdABCD* operon from *Azoarcus* sp. (21) (see Fig. S4 in the supplemental material). Therefore, the *A. fulgidus* multisubunit Mo-containing enzyme could be involved in the hydroxylation of the double bond of alk-1-enes, but such a mechanism still needs to be demonstrated.

Based on physiologic and metabolic results, we here provide direct evidence that *A. fulgidus* is able to grow on mid- to long-chain fatty acids and to form linear fatty acids from *n*-alk-1-enes, although the complete metabolic pathways of alkene degradation by *A. fulgidus* still need to be further characterized.

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